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14. ABSTRACT Increased solar radiation and other unknown factors induce excess melanin production in melanocytes accumulated in localized areas of the skin, leading to formation of benign nevi and infrequently, dysplastic nevi that may progress to melanoma. I hypothesized that excess melanin production in melanocytes of such nevi may cause physico-chemical constraints on the metabolic activities of DNA and RNA, which, in rare instances, may induce pro-survival responses, including oncogenic mutations, in cellular DNA. This may lead to selective transcription and/or translation that support oncogenic transformation of one or more of these cells. To test this hypothesis, long-term production of excess melanin in melanocytes derived from fair-skinned and dark-skinned individuals was induced with chronic tyrosine exposure or chronic UV-radiation treatment. Long-term melanin production in cells from fair-skinned individuals caused a small increase in their proliferation. Chronic melanin production over a period of six months revealed unique gene expression patterns associated with growth promoting signaling networks in melanocytes from fair-skinned individuals. Functional cell biological analyses revealed that these effects resulted in increased proliferation and transformative potential of the melanocytes derived from fair-skinned, but not dark-skinned individuals. This study suggests that melanin pigment may play an important role in the oncogenic transformation of melanocytes from fair-skinned individuals.					
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Introduction

UV radiation is a major occupational risk factor of melanoma for the U.S. combat soldiers. Melanin pigment is the primary defense in the skin and eyes against the mutagenic effects of UV radiation. Melanin forms an envelope over the nucleus and prevents UV-radiation damage to nuclear DNA. However, many melanoma tumors originate from melanocytes within highly pigmented nevi. In such cases, UV-damaged melanin is thought to produce mutagenic reactive oxygen species¹, which may contribute to oncogenesis. But there is no UV-radiation damage in melanomas that develop from non-sun-exposed areas of the body like mucous membranes or the soles of feet. In these instances, it is compelling to question the role of undamaged melanin in oncogenesis. To answer this question, it is imperative to separate the UV-protective role of melanin from its other cellular activities, and determine the effects of excess melanin in melanocytes from a conceptually different perspective. The current study is focused on exploring the oncogenic potential of excess melanin production in melanocytes in the presence and absence of UV damage. This study has the potential to impact novel therapeutic strategies against melanoma, and the formulation of melanoma risk prevention strategies for combat soldiers in the U.S. army.

Melanin is synthesized in melanocytes and transferred to other cell types like keratinocytes to provide a homogeneous pigmentation in the skin. The enzyme tyrosinase is the rate limiting factor in the enzymatic conversion of the amino acid tyrosine into melanin. Melanin is stored in melanosomes in the cytoplasm and is also present in the nucleus, although its nuclear functions are unknown. Melanin itself seems to promote cellular differentiation, which may be a contextual role, since this role seems to be promoted by the oncogenic MITF transcription factor^{2, 3}. It has been suggested that in its UV-protective role, melanin forms an envelope over the nucleus and DNA. This mechanism may also protect DNA from genotoxic/cytotoxic drugs, either through direct sequestration of the drugs or by acting as a physical barrier that prevents access to the DNA^{4, 5}. Similarly, it is possible that excess melanin in cells that constitute benign nevi may cause physico-chemical constraints on DNA and RNA metabolic activities, such as transcription and translation. In rare instances, this may induce pro-survival responses, including mutations in cellular DNA. This may lead to selective transcription and/or translation that support oncogenic transformation of these cells. **I hypothesized that excess melanin production may induce a pro-survival response in cells, leading to selective transcriptional regulation that supports oncogenic transformation of melanocytes.** To test this hypothesis, I proposed the following two specific aims.

Aim 1- Determine transcriptional changes in melanocytes after inducing excess melanin production.

Aim 2- Determine the transformation potential of excess melanin production in melanocytes.

Body

For executing the above specific aims, the following tasks were performed. The progress of these tasks are described under separate headings for the previously submitted final report (February 2010 – January 2011) and the current addendum (February 2011 - July 2011).

Task 1A: Induce melanin production in melanocytes and perform UV-radiation treatments.

FINAL REPORT: Two sets of low melanin containing primary neonatal melanocytes (LP1 and LP2) from two distinct individuals and two sets of high melanin containing primary neonatal melanocytes (HP1 and HP2) were obtained from Lonza/Invitrogen (Carlsbad, CA). These LP and HP sets represent melanocyte cells from fair-skinned Caucasian and dark-skinned African racial backgrounds. Primary adult melanocytes from Lonza and ATCC (Manassas, VA) were also obtained. However, the adult

melanocytes were overly sensitive to the treatments in this study, and were not pursued further. All primary melanocytes in this study were grown in 254 media with HMGS-2 serum supplement in 10 cm dishes. Long-term chemical induction of melanin synthesis in these cells was performed by inclusion of 0.5 mM of L-tyrosine in the cell culture medium. At this dose, L-tyrosine induced high levels of pigmentation in these cells, without affecting their short-term growth (Figure 1A). UV-radiation induction of melanin synthesis was performed as described⁶ by daily treatments of the cells using an FS-40 sunlamp (National Biological, Twinsburg, OH). This lamp emits a continuous spectrum of UV radiation from 270 to 390 nm, with a peak emission at 313 nm; approximately 65% of the radiation is within the UVB (290-320 nm) range. The UVB output of this bulb averaged 1.35 J/m²/sec, at a lamp to target distance of 23 cm as measured with an IL-1700 radiometer (International Light, Newburyport, MA). Approximately 1 X 10⁶ melanocytes were plated in 10 cm tissue culture dishes, the medium was removed and the cells were re-suspended in PBS. The cells were exposed to 50 J/m² dose of UVB radiation. The PBS was removed and the cells were then re-suspended in tissue culture medium. Different non-erythema doses of UVB were tested on the cells initially. The dose of 50 J/m² induced the highest levels of melanin over long periods of time, with no apparent toxicity to the primary melanocytes (Figure 1B). For quantifying melanin, cell pellets from 10⁶ cells/sample were collected by centrifugation. The cell pellets were suspended in 1 ml of 1 N NaOH solution and incubated at 80⁰ C for 2 h. The resulting cell lysates were centrifuged at 6000 rpm for 5 min. The pellet containing DNA and other cell debris was discarded, the optical absorbance of the clarified lysates at 470nm wavelength was measured, and melanin content was quantified against a standard curve generated using synthetic melanin (Sigma, St. Louis, MO). Long-term melanin induction treatments were carried out for a period of 6 months. For the long-term chemical induction, tyrosine containing media was replenished every 3 days, while the UV-radiation treatments were performed every day. The daily UV-radiation treatments for 6 months emulated the occupational exposure of active military personnel in tropical regions. Melanin levels were quantified every 15 days. After completion of the incubations, one set of plates was used for cell lysate preparation in RIPA lysis buffer and stored in -80 °C freezer for future protein analysis. A second set was used for RNA isolation. Task 1A has been successfully accomplished.

CURRENT ADDENDUM: None. Task 1 was fully accomplished in the period of the final report.

Task 1B: Gene expression profiling to detect melanin-induced transcriptional changes.

FINAL REPORT: Total cellular RNAs from the melanocytes in the experiments in Task 1A were purified using the RNeasy kit (Qiagen, Valencia, CA). RNA quality control (RNA Integrity Number determination) was performed on an Agilent Analyzer. Whole genome expression profiling was performed using the Illumina human HT12 beadchip microarray, which contains over 48,000 unique probes representing 25,000 annotated genes, gene candidates and splice variants per array (derived from the RefSeq and UniGene databases). The data was background corrected, normalized and analysis of gene expression networks was performed using Ingenuity Pathway Analysis (IPA) (www.ingenuity.com).

IPA signaling network analysis of the long-term UV-radiation treated cells showed that p53 signaling network was most significantly dysregulated in the low pigmented melanocytes, LP1 and LP2 (Figure 2A). The p53 regulatory components like p21/Waf1 were significantly upregulated in these cells. Other major signaling networks affected in the LP cells upon melanin induction by UV were cell cycle regulatory pathways and cancer tissue response factors (Figure 2A). In the high pigmented melanocytes (HP1 and HP2), oxidative phosphorylation, which is the final phase of aerobic respiration, was most dysregulated by long-term UV-radiation treatment (Figure 2B). This involved downregulation of the entire program of oxidative phosphorylation genes. This result is surprising, since cellular metabolic regulation by long-term melanin induction has not been observed before. Other major dysregulated networks in the melanin-induced HP cells were downregulation of protein ubiquitination pathways and upregulation of mitotic regulatory Polo like Kinases (PLK) (Figure 2B). Interestingly, expression of the melanin

biosynthetic machinery like the POMC gene, which translates into α MSH, β MSH, and γ MSH peptides, tyrosinase (TYR) and tyrosinase related protein (TYRP1) genes was slightly downregulated by the long-term melanin induction by UV (Figure 2C). Expression of microphthalmia associated transcription factor (MITF) and dopachrome tautomerase (DCT) was downregulated, while the expression of the melanocortin 1 receptor gene (MC1R) was upregulated in the LP cells and to a greater extent in the HP cells (Figure 2C).

CURRENT ADDENDUM: Gene expression network analysis of the long-term tyrosine treated cells was performed using IPA. The genes included in the IPA analysis had cutoffs of >2-fold change in gene expression compared to control treatments, and $P < 0.01$. This analysis showed upregulation of cell cycle component genes in the LP1 and LP2 cells, followed by cancer tissue functions and cellular glycolysis (Figure 3A). The p53 signaling network was not significantly altered in the melanin-treated LP1 and LP2 cells, unlike the UV-treated cells (Figure 3A). In the HP1 and HP2 cells, oxidative phosphorylation and protein ubiquitination were downregulated with melanin treatment, which was similar to the UV-radiation treatment (Figure 3B). The PLK network was however not altered in the tyrosine-treated HP cells, unlike the UV-treated HP cells (Figure 3B). The melanin biosynthetic machinery genes were altered with the tyrosine treatment, which was similar to their alterations with the UV-radiation treatment (Figure 3C). This suggests that except the p53 and glycolysis networks, other major effects of melanin induction by UV-radiation and tyrosine treatments were similar in the LP cells. Similarly, except the PLK and cytoskeletal function networks in the HP cells, other major effects of melanin induction by the two different methods were similar in both types of cells. Task 1B has been successfully accomplished.

Discussion: Induction of excess melanin biosynthesis by two alternate methods- UV-radiation treatment and tyrosine treatment, resulted in unique gene expression changes in the lightly pigmented melanocytes (LP cells) derived from fair-skinned individuals of Caucasian descent versus heavily pigmented melanocytes (HP cells) derived from dark-skinned individuals of African descent. Primary among these changes after UV-radiation treatment are activation of p53 pathway, followed by activation of cell cycle functions and cancer tissue functions in the LP cells. In the HP cells, the UV treatment resulted in downregulation of oxidative phosphorylation and protein ubiquitination functions and upregulation of PLK functions. Interestingly, melanin induction by tyrosine-treatment did not alter the p53 pathway in the LP cells, suggesting that activation of p53 pathway by UV-radiation treatment is not related to induction of melanin synthesis. However, cell cycle components and cancer tissue functions were upregulated by melanin induction with tyrosine, suggesting that these two functions are related to melanin induction, by either method. Tyrosine treatment caused an upregulation of cellular glycolytic genes in the LP cells, which was not observed with the UV-treatment, suggesting that this effect was not related to melanin synthesis. In the HP cells, tyrosine treatment caused a downregulation of oxidative phosphorylation and protein ubiquitination, just like the UV-treatments, but it did not increase PLK functions, suggesting that only the former two effects are related to melanin synthesis.

Task 1C: Validation of relevant gene expression changes identified by gene expression profiling.

FINAL REPORT: To determine if the above observed changes in gene expression are a result of melanin induction by the cells, and not independent cellular responses to the effects of UV-radiation or tyrosine treatments on gene expression, the melanin biosynthesis pathway was inactivated in the cells prior to their long-term treatment with UV-radiation or tyrosine. For this purpose, transient siRNA knockdown of individual enzymes that are specifically involved in melanin biosynthesis– phenylalanine hydroxylase, tyrosinase, tyrosine hydroxylase and dopachrome tautomerase was performed. Knockdown of the rate-limiting tyrosinase enzyme led to the best reduction in melanin levels in the primary melanocytes, as well as melanotic melanoma cells (Figure 4A). The pSilencer 5 Retro System (Ambion,

Austin, TX) was used for generating tyrosinase shRNA vectors. However, this retroviral system could not induce stable knockdown of tyrosinase in the primary melanocytes, although it did so in melanoma cells. The relatively slow cell division rate of primary melanocytes, apart from other unique characteristics may have prevented efficient retroviral delivery and shRNA expression in these cells. After several failed attempts with the pSilencer Retro system, the viral delivery system was switched to the pGIPZ lentiviral system from Open Biosystems, Huntsville, AL, (Figure 4B) to generate stable tyrosinase shRNA expression in the melanocytes. A non-targeting shRNA (shCON) was included as a control for all stable transductions. Transduction of the melanocytes with the pGIPZ lentivirus containing a cloned-in tyrosinase shRNA resulted in stable knockdown of >70% melanin levels in the LP & HP cells (Figure 4C). This however had significantly delayed the timeline for the progress of this project. Hence, I had requested the EWOFF for 6 months, which has enabled me to complete this research in its entirety.

CURRENT ADDENDUM: After successfully achieving stable transduction of the melanocytes with the tyrosinase shRNA using the pGIPZ lentiviral system, the tyrosinase knocked-down cells were treated with UV-radiation or tyrosine over a period of six months as described before. Melanocytes expressing a non-targeting shRNA vector were included as controls. The stable expression of tyrosinase shRNA or control shRNA was maintained by inclusion of 0.5 μ g/ml puromycin in the culture medium. However, the shRNA transduced cells did not survive the UV-radiation treatments. This could possibly be due to increased UV-damage in absence of melanin or a combination effect of UV-damage and presence of puromycin in the culture media. On the other hand, the shRNA transduced LP cells survived the six months of tyrosine treatment, and exhibited consistent knockdown of the melanin levels (Figure 5A). In the HP cells however, the knockdown of melanin could not be maintained over the six month time frame. This could possibly have been due to disruption of the cytoplasmic shRNA-DICER machinery or the downstream RISC machinery by excessively high levels of melanin in the HP cells.

After conclusion of the tyrosine treatments, gene expression microarray analysis was performed using RNA extracted from the LP cells. IPA analysis showed upregulation of cell cycle genes and cancer tissue factor genes in the control LP cells, but not in the tyrosinase shRNA expressing LP cells (Figure 5B). However, the tyrosine-treatment induced upregulation of glycolysis genes was not impacted in the tyrosinase shRNA expressing LP cells (Figure 5B). This strongly suggests that upregulation of cell cycle and cancer tissue factor functions by tyrosine treatment were in fact related to increased levels of melanin in the cells. Task 1C has been successfully accomplished with two exceptions- **1.** The effects of melanin induction by UV-radiation could not be validated due to premature cell death of the melanin knocked-down cells before completion of the six months UV treatment. **2.** The knockdown of melanin levels were not sustained over a six month period in the HP cells, which has limited our subsequent analysis to the LP cells only.

Discussion: The functional validation study in the tyrosinase knocked-down LP cells showed that treatment with tyrosine failed to upregulate cell cycle and cancer tissue factor genes. This suggests that activation of these genes was dependent on excess melanin synthesis. However, glycolytic genes were not upregulated in the stable cells with reduced melanin, suggesting that these genes were dependent on melanin-independent effects of tyrosine on the cells. An interesting finding of this study is that except the gene encoding MC1R receptor, none of the other genes involved in melanin biosynthesis were upregulated by the long-term production of excess melanin. In fact, MITF and DCT genes were potently downregulated, and the rate limiting, melanin synthetic enzyme Tyrosinase, was slightly downregulated. Validation of the same was provided by the LP cells with abrogated melanin production, which did not show these gene expression changes. These results suggest that the LP cells possess control mechanisms that may prevent excessive synthesis of melanin. MC1R may be critically regulated by melanogenic stimuli like UV radiation or other environmental factors. MC1R in turn may induce post-translational activation or stability of the melanin biosynthetic machinery to increase melanin production in melanocytes. Also, the levels of upregulation of MC1R were different between the LP and HP

melanocytes, suggesting that melanogenic stimuli may not similarly activate MC1R in the melanocytes of individuals from the two different racial backgrounds. Many Caucasian populations with low melanogenic responses have sequence variations in the MC1R gene that reduce its activity⁷. A similar variation in the LP melanocytes may be responsible for lower melanogenic responses in these cells.

Task 2A: Determination of the transformation potential of melanin producing cells.

FINAL REPORT:

Changes in Cell Cycle: To determine if the UV-radiation- and tyrosine-induced production of excess melanin results in changes in cell growth and division, cell cycle analysis of the melanin-induced melanocytes from Task 1A was performed using a propidium iodide dye-based, flow cytometry assay. After 6 months of treatments, the cells were seeded in 6-well plates and grown for seven days, and cell cycle analysis was performed. While no significant changes were observed, both treatments induced a small reduction in the G1 phase of the LP cells, and a small increase of the G1 phase in the HP cells (Figure 6A).

Changes in Proliferation: Cell proliferation over a period of one week was measured using the CellTiter-Blue cell proliferation assay (Promega, Madison, WI). A small increase in proliferation was observed in the LP cells that were induced to produce excess melanin by tyrosine treatment and UV-radiation-treatment (Figure 6B). However, no significant changes in cell proliferation were observed in the similarly treated HP cells (Figure 6B).

Cellular Transformation Assays: To determine the migration potential of the excess melanin producing cells, a scratch assay was performed on the LP cells growing in 10 cm dishes, and the migration of cells into a cleared area was photographically documented. The cells that were induced to synthesize melanin by UV-radiation or tyrosine treatment for six months showed a higher rate of migration over a period of 2 weeks. Migration of the tyrosine treated cells was slightly reduced compared to the UV-radiation treated cells (Figure 6C). To determine the transformative potential of the excess melanin producing melanocytes, foci formation of the differentially treated melanocytes was performed using an anchorage-independent soft agar assay. Melanin induction by tyrosine treatment of the melanocytes slightly increased foci formation by the LP cells, and increased the size of the foci in the UV-treated cells (Figure 6D). This suggests oncogenic transformative potential of melanin induction in the melanocytes. Task 2A has been successfully accomplished.

CURRENT ADDENDUM: No further work was necessary since Task 2A had been accomplished.

Task 2B: Validation of the transformation potential.

FINAL REPORT: No validation experiments were performed since stable shRNA expressing melanocytes were under development during this period.

CURRENT ADDENDUM: The transformation potential of long-term excess melanin-production was validated using the newly developed stable melanocytes expressing tyrosinase shRNA and control shRNA. To determine if the changes in cell migration and transformation observed in task 2A are a result of excess melanin accumulation by the cells, and not melanin-independent cellular responses to tyrosine treatments, the cytological studies were performed using the melanocytes expressing stable shRNA against tyrosinase and the control shRNA.

Changes in Cell Cycle: After six months of tyrosine treatments, the LP cells that stably express shCon or shTyr were seeded in 6-well plates, grown for seven days, and subjected to cell cycle analysis. The results showed that except a small increase of G1 phase cells and a small decrease of S phase cells in the shTYR cells, no significant differences were observed between the shCon and shTyr expressing cells (Figure 7A).

Changes in Proliferation: After treating the stably transduced LP cells with tyrosine for six months, the cells were seeded in a 96 well plate at a density of 10^3 cells/well. Cell proliferation assay was performed

using CellTiter-Blue reagent after 7 days of incubation. The results showed that the tyrosine treatments increased the proliferation of the control cells, but not the tyrosinase shRNA expressing cells (Figure 7B). **Cellular Transformation Assays:** To get a quantitative profile of the migration of the cells, cell migration assay was performed using an Xcelligence DP migration assay, which is based on electrical impedance technology (Roche Applied Science, Indianapolis, IA). After the six months of growth in presence of tyrosine, 10^5 cells in serum-free and growth-factor free media were plated in individual well inserts of the Xcelligence impedance plates. 5% HMGS2 growth factor containing media was used as a chemo-attractant for migration of the cells. Cell migration over a period of 24 h was monitored. While the control LP cells showed increased migration, the tyrosinase shRNA cells did not do so (Figure 7B). When the soft-agar foci formation assay was performed using the stable transfected cells, neither the control cells nor the shRNA expressing cells could form foci. This may possibly be due to the extra stress induced by puromycin present in the media. Task 2B has been successfully accomplished, with one exception, which is the same as in Task 1B- the effects of melanin induction by UV-radiation could not be validated due to premature death of the melanin knocked-down cells before completion of the UV-treatment schedule.

Discussion: As suggested by the cell proliferation assays, migration assays and soft-agar foci formation assays, long-term synthesis of excess melanin increased the proliferation and transformative potential of the LP melanocytes. Validation for this was demonstrated by the functional study using the tyrosinase shRNA-expressing LP cells. These cells did not show increased proliferation or migration, unlike the control cells, suggesting that the absence of excess melanin synthesis prevented transformation of the LP cells.

Task 2C: Manuscript preparation, submission and meeting presentation.

FINAL REPORT: None.

CURRENT ADDENDUM: A manuscript detailing the findings of this work is currently under preparation for publication. These findings will also be presented as a poster or talk in an international melanoma and skin cancer meeting in 2012, upon completion of additional work outside the scope of this report.

Key Research Accomplishments

- Excess melanin synthesis over extended periods caused unique gene expression changes in the lightly pigmented melanocytes (LP cells) derived from fair-skinned individuals of Caucasian descent versus heavily pigmented melanocytes (HP cells) derived from dark-skinned individuals of African descent.
- Long-term synthesis of excess melanin resulted in the induction of cell cycle activation genes and cancer tissue function genes in the LP cells, but not the HP cells. In the HP cells, excess melanin synthesis resulted in a decrease of oxidative phosphorylation genes and protein ubiquitination genes.
- Functional validation of these findings was performed by stable disruption of melanin biosynthetic machinery using stably transduced shRNA against tyrosinase, followed by induction of melanin synthesis. Knockdown of tyrosinase enzyme by stable shRNA transfection resulted in >70% reduction of melanin levels even after induction with tyrosine for six months, with a concomitant abrogation of the gene expression effects associated with the long-term melanin induction.

- Long term synthesis of excess melanin by the normal LP melanocytes increased their proliferation and oncogenic transformative potential, to the extent that these cells are now immortal. Unlike the LP cells, the HP melanocytes did not exhibit a transformative potential.

Reportable Outcomes

- This study made a novel discovery that excess melanin increases the proliferation and oncogenic transformation of Caucasian melanocytes. These results are currently put together for publication in a high impact journal.
- The results of this study will be presented as a talk or poster in an international melanoma and skin cancer meeting in 2012.
- The results from this study will serve as preliminary evidence to apply for larger grants in the scale of NIH R01, for further understanding and validating the role of melanin in human melanoma pathobiology.

Conclusions

- Chronic production of excess melanin caused unique gene expression changes in lightly pigmented melanocytes derived from fair-skinned individuals of Caucasian origin compared to highly pigmented melanocytes derived from dark-skinned individuals of African origin.
- Chronic melanin induction increased the proliferation and oncogenic transformative potential of melanocytes from Caucasian origin, but not African origin. These results however should be interpreted with caution since they reflect *in vitro* effects in a small sample size.
- A long-term study with animal models and human patient specimens is needed to determine if excess melanin-induced changes in gene expression are indeed responsible for melanocyte proliferation and oncogenesis. Such a study could identify novel epidemiological factors for melanoma, apart from UV-radiation. This will have a direct impact on the development of melanoma prevention strategies.

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Figures

Figure 1A

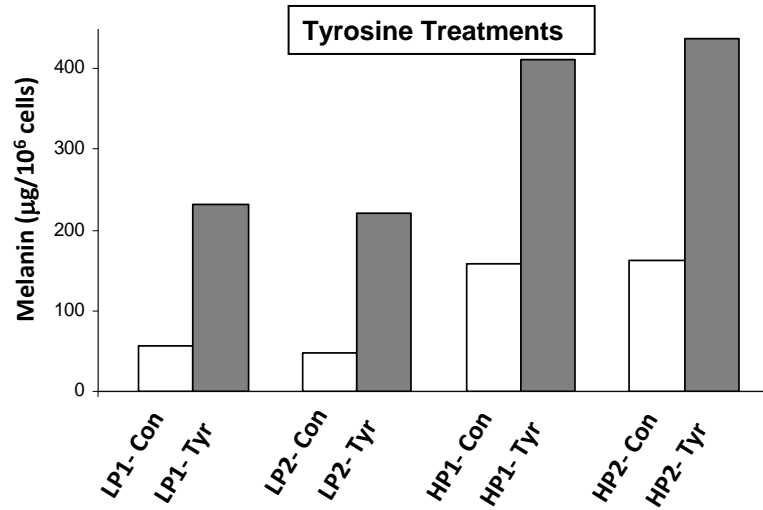


Figure 1B

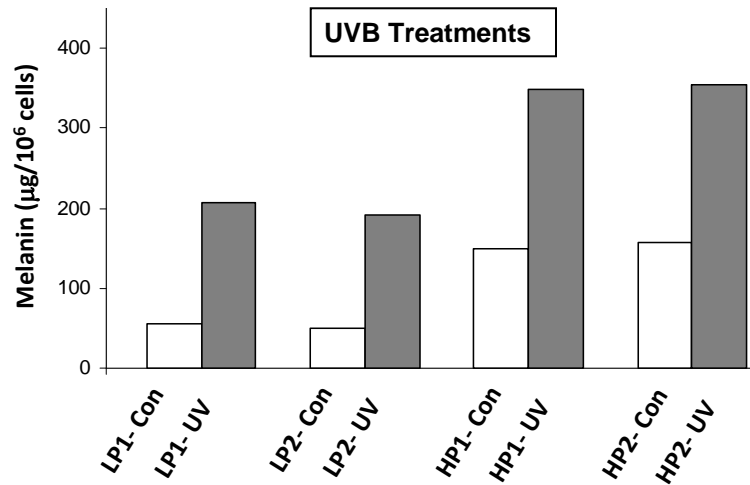


Figure 1: (A & B) The low pigmented melanocytes (LP1 & LP2) and the high pigmented melanocytes (HP1 & HP2) were treated with tyrosine or UVB radiation for a period of 6 months. For the tyrosine treatments, the cells were replenished with 0.5mM tyrosine (Tyr) containing media every 3 days, and for the UV radiation treatments, the cells were treated with 50J/m² UVB radiation (UV) every day, as described in Task1 A. Control (Con) cells were grown alongside the treated cells and underwent similar changes in media, without tyrosine or UVB treatments. Melanin levels at the end of the treatment period were spectrophotometrically quantified. The tyrosine treatment induced higher levels of melanin in the cells compared to UV radiation. HP cells showed a higher accumulation of melanin compared to the LP cells. Data is representative of triplicate samples. x-axis, cells and treatments. Y-axis, concentration of melanin in micrograms per million cells.

Figure 2A

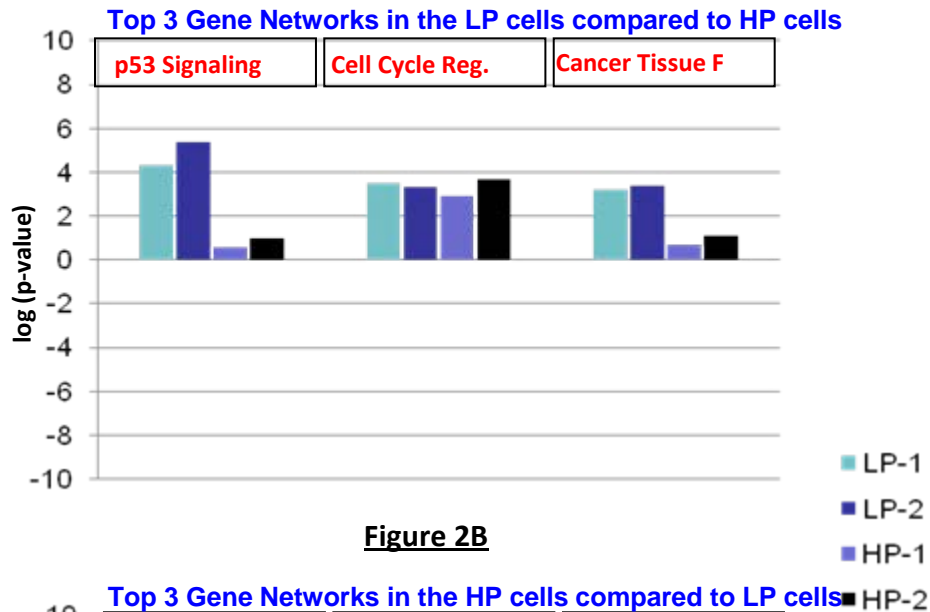


Figure 2B

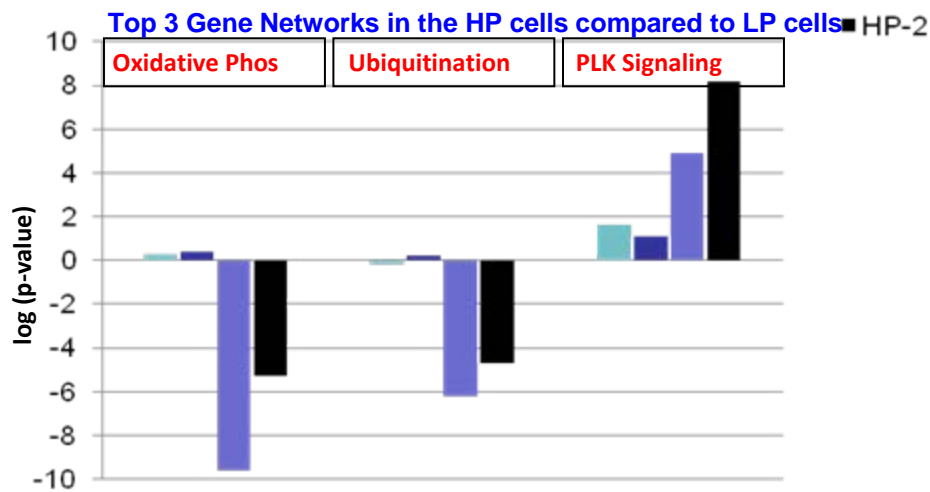


Figure 2C

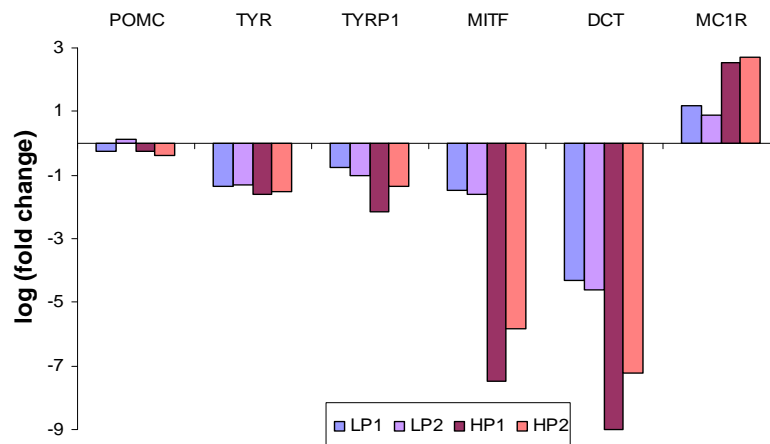


Figure 2: (A & B) After 6 months of daily UV-radiation treatments, the cells were incubated for 72 h and total RNA was extracted from the UV-treated and control cells, and a whole genome expression analysis was performed using Illumina HT12 bead-chips. The data was normalized, log-transformed and a gene expression network analysis was performed using Ingenuity Pathway Analysis (IPA). The data showed that p53 signaling was the most dysregulated network in the UV-treated LP cells, compared to the HP cells, while Oxidative phosphorylation was the most dysregulated network in the UV-treated HP cells compared to the LP cells. Interestingly all the oxidative phosphorylation genes in the HP cells were downregulated, while the p53 signaling genes were upregulated in the LP cells.

(C) Long-term UV radiation caused a decrease in the expression of the tyrosine biosynthetic enzymes, with the exception of MC1R in the LP and HP cells. Some of these changes were more pronounced in the HP cells. Data represents log fold change ratio of treated cells versus control cells.

Figure 3A

Top 3 Gene Networks in the LP cells compared to HP cells

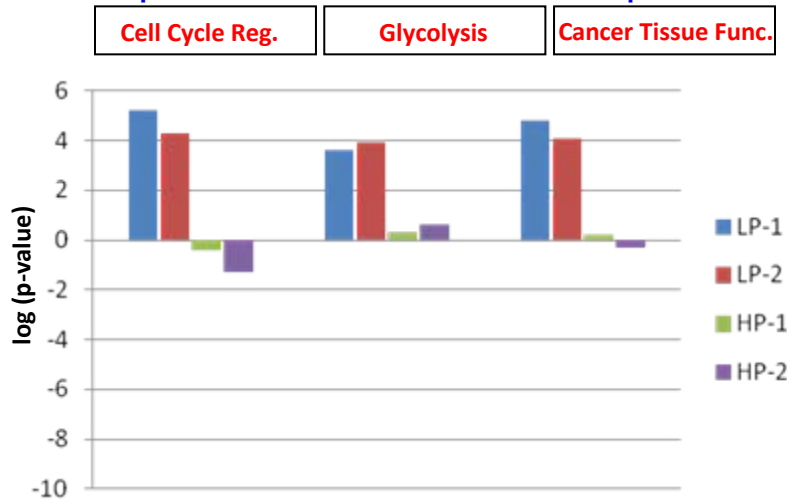


Figure 3B

Top 3 Gene Networks in the HP cells compared to LP cells

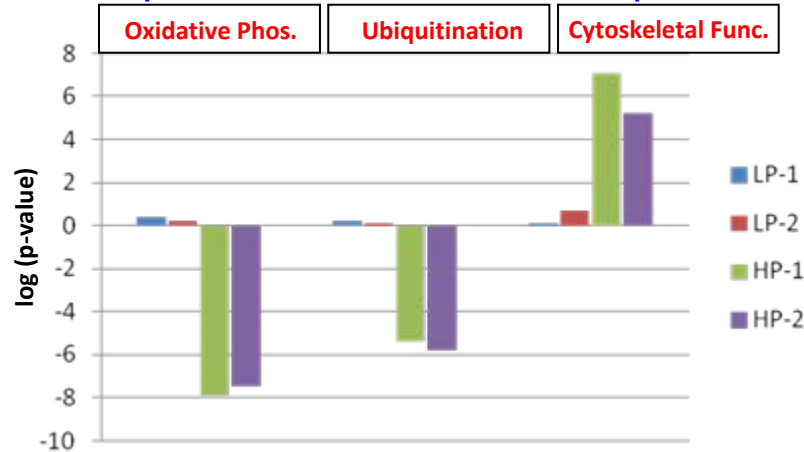


Figure 3C

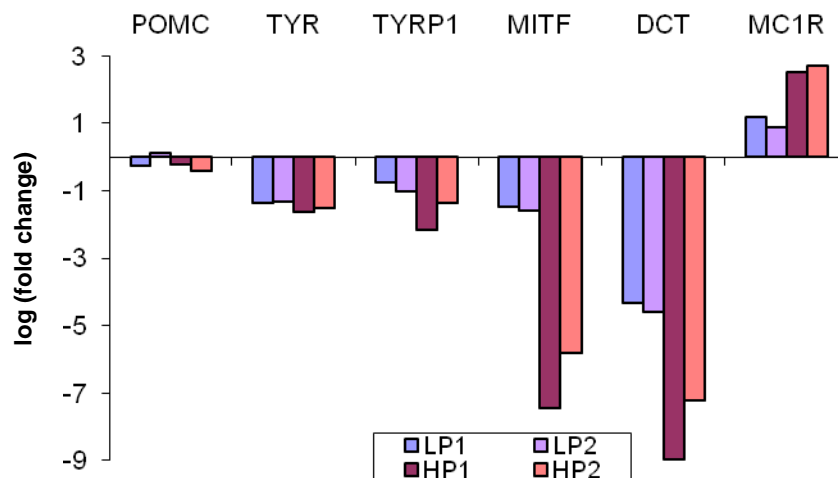


Figure 3: (A, B & C) Similar experiments as in Figure 2 A, B & C, but with 6 months of continuous treatment with tyrosine, instead of UV- radiation. After 6 months of continuous exposure to tyrosine in the growth media, with changes of tyrosine containing media every three days, total RNA was extracted from the tyrosine-treated and control cells, and a whole genome expression analysis was performed. The data was normalized, log transformed and a gene expression network analysis was performed using Ingenuity Pathway Analysis (IPA). In figures A and B, the data resembles the UV-radiation treatments, except glycolysis in figure A and cytoskeletal functions in figure B. The gene expression changes in melanin biosynthetic genes in figure C are similar to those with UV-radiation treatment.

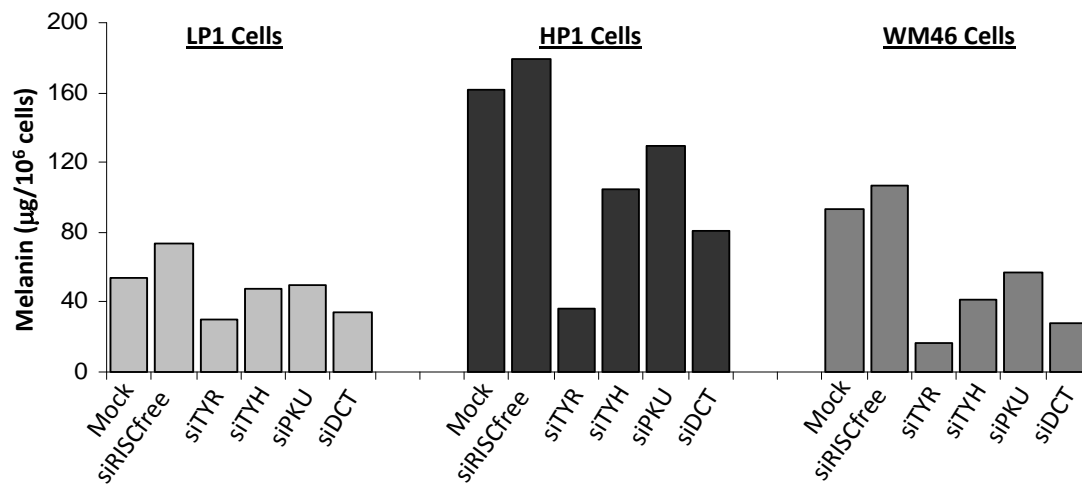
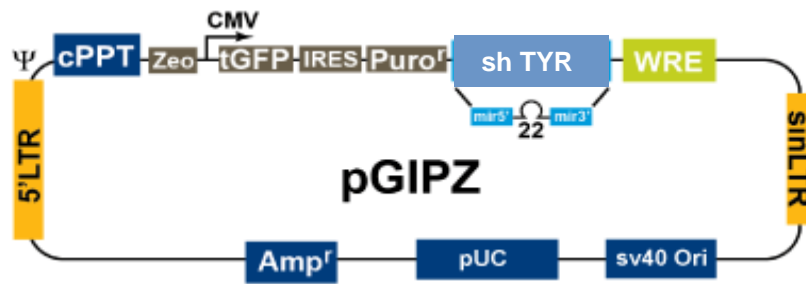
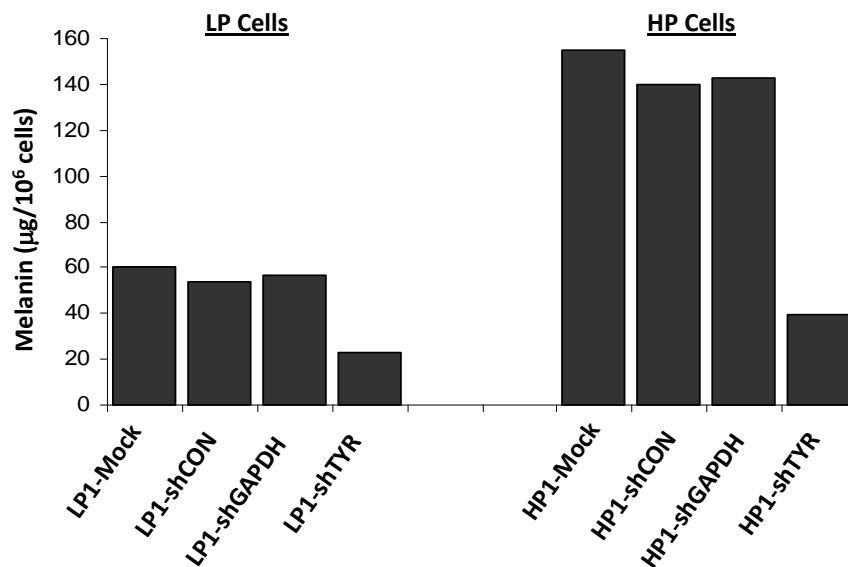
Figure 4A**Figure 4B****Figure 4C**

Figure 4: (A) The LP1, HP1 cells and WM46 melanotic melanoma cells were transfected with siRNAs directed against melanin biosynthetic enzymes, tyrosinase (siTYR), tyrosine hydroxylase (TYH), phenylalanine hydroxylase (siPKU), and dopachrome tautomerase (siDCT). The cells were incubated for 96 h and harvested for melanin quantitation. The siRISCfree siRNA was used as a control, which slightly increased melanin levels in the cells. All siRNAs directed against the melanin biosynthetic enzymes reduced the amount of melanin in varying amounts. The tyrosinase siRNA was most Effective, and consistently reduced melanin levels in all cells. **(B)** Tyrosinase shRNA sequence was inserted into the pGIPZ lentiviral vector containing a CMV polymerase II promoter and a WRE sequence for efficient shRNA processing. **(C)** The LP1 and HP1 cells were transduced with virus containing empty vector (shCON), GAPDH (shGAPDH) or tyrosinase (shTYR) shRNAs. One month after transduction, puromycin selected cells were quantified for melanin. The data shows efficient (>70%) knockdown of melanin synthesis in the LP and HP cells.

Figure 5A

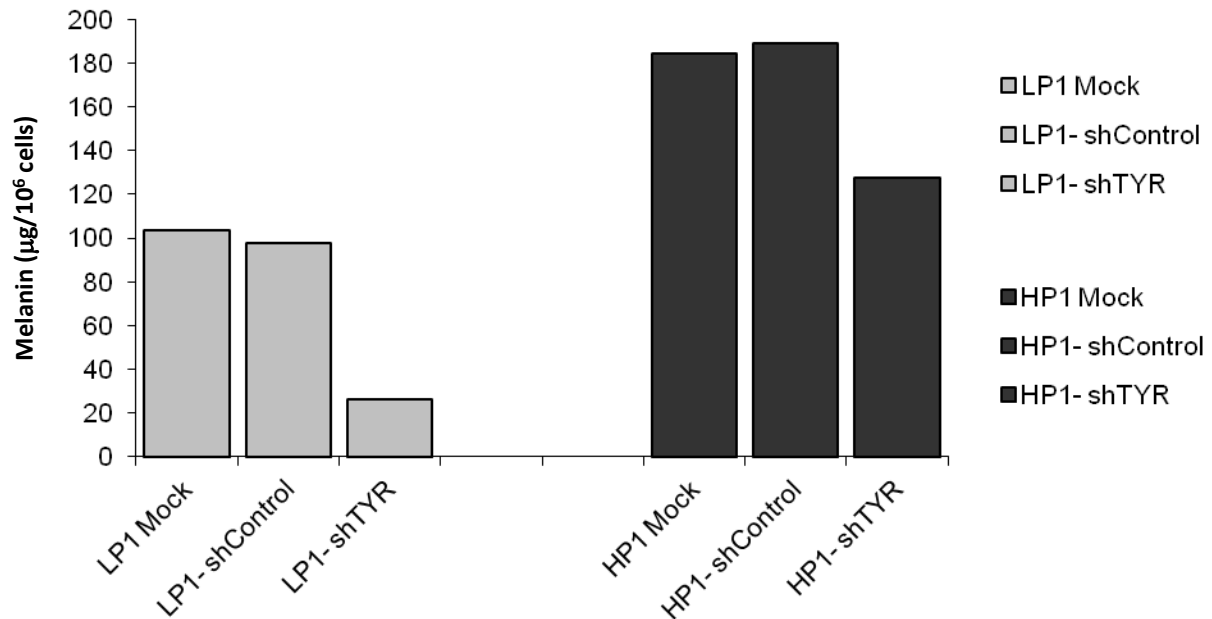


Figure 5B

Top 3 Gene Networks in the Controls versus shTyr transduced LP cells

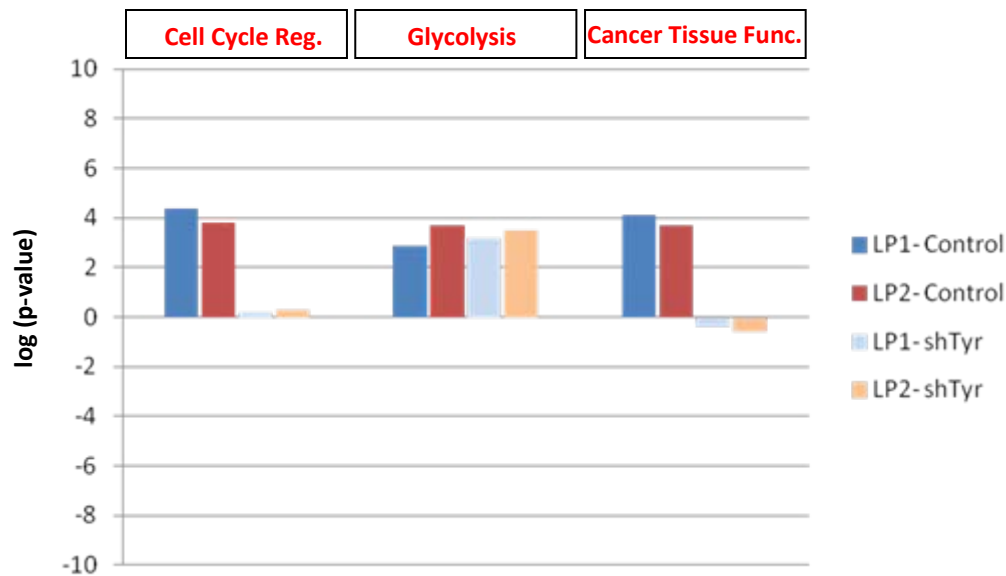


Figure 5: (A) Stable transduction of the LP1 and HP1 cells was performed with pGIPZ virus containing empty vector (shControl), or tyrosinase (shTYR) shRNAs. These cells were exposed to tyrosine and puromycin containing media for six months. The untransduced cells (Mock) were also similarly treated with tyrosine along with the transduced cells. Melanin was quantified and the data was plotted. The data shows efficient (>70%) knockdown of melanin synthesis in the LP cells but <50% knockdown in the HP cells after 6 months of continuous tyrosine exposure. Similar results were obtained for the LP2 and HP2 cells. **(B)** RNA was extracted from the shControl and shTYR transduced cells, and gene expression microarray analysis was performed, followed by IPA analysis, as described before. The data shows that downregulation of melanin synthesis by shTYR in the LP cells (LP1-shTyr And LP2-ShTyr) resulted in abrogation of the Cell cycle regulatory genes and Cancer tissue factor function genes, but not the Glycolysis genes.

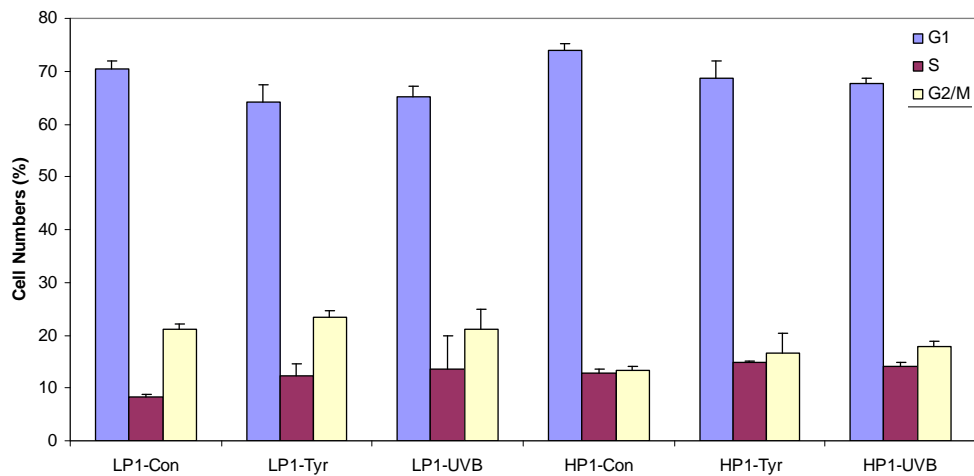
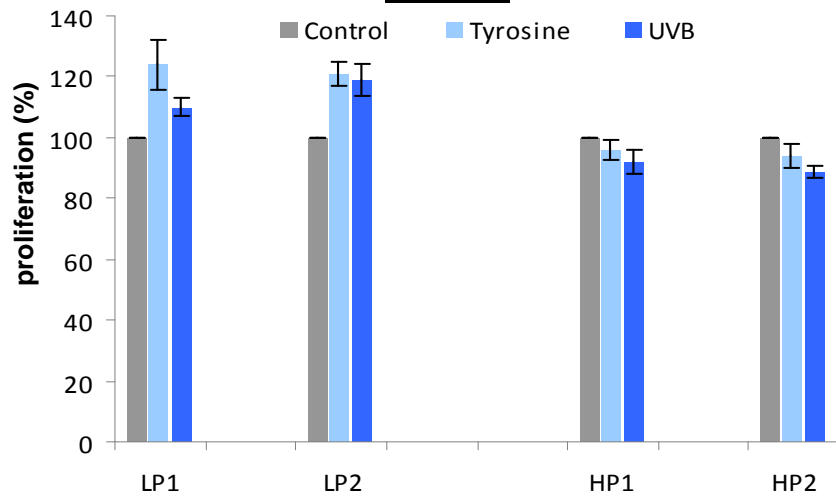
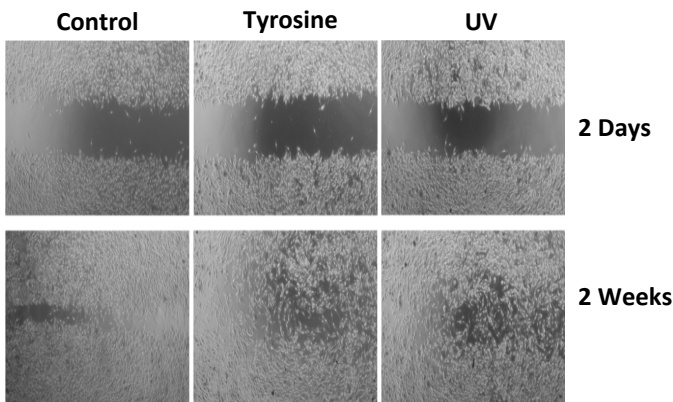
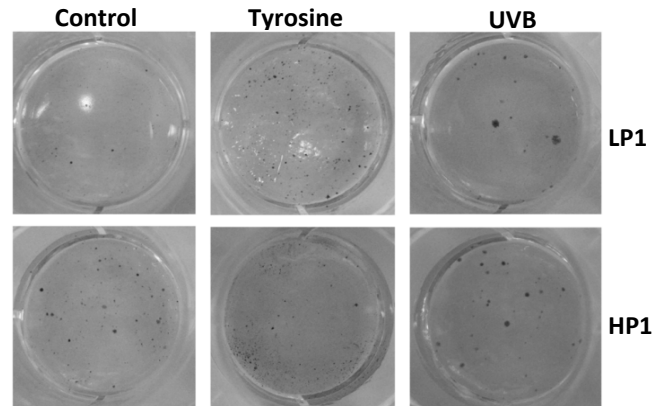
Figure 6A**Figure 6B****Figure 6C****Figure 6D**

Figure 6: (A) After 6 months of tyrosine or UV-radiation treatments, one set of the LP and HP cells were seeded in 6-well plates at a density of 10^5 cells/well and incubated for 7 days. The cells were then harvested, fixed in 70% ethanol, stained with propidium iodide, and cell cycle analysis was performed. The % of cells in G1, S and G2/M phases of the cell cycle are indicated. (B) The LP and HP cells with similar treatments as in (A) were seeded in 96-well plates at a density of 1000 cells/well and incubated for 7 days, after which the CellTiter-Blue proliferation assay was performed. The data showed that tyrosine and UV treatments increased the proliferation of the LP cells, but not the HP cells. (C) A cell migration "scratch" assay with the LP cells showed that the 6 month tyrosine and UV treatments slightly increased the migration rate of the cells. (D) A soft agar colony formation assay showed that the untreated LP cells formed fewer colonies compared to the HP cells, with a mean diameter of 0.5 mm per colony. Tyrosine treatment increased the number of colonies, but not the mean size of the colonies in the LP cells. In the HP cells, there was no significant change in the number or size of the colonies in the tyrosine treated cells. UV treatment however increased the mean size of the colonies in the LP cells to 3 mm, but only about 1 mm in the HP cells.

Figure 7A

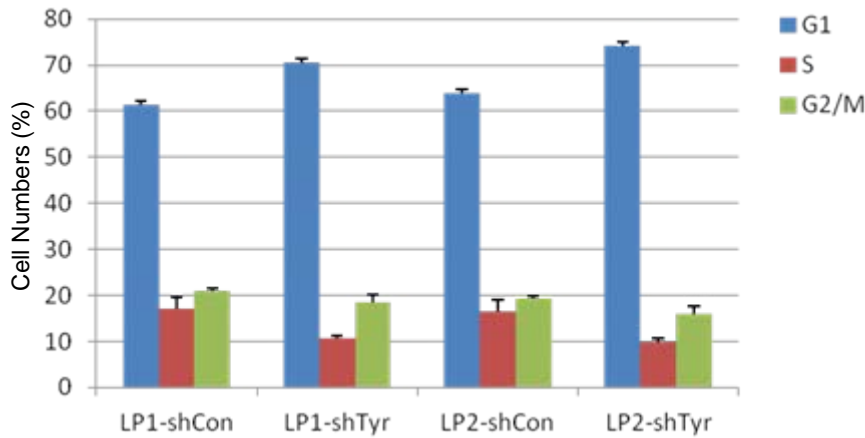


Figure 7B

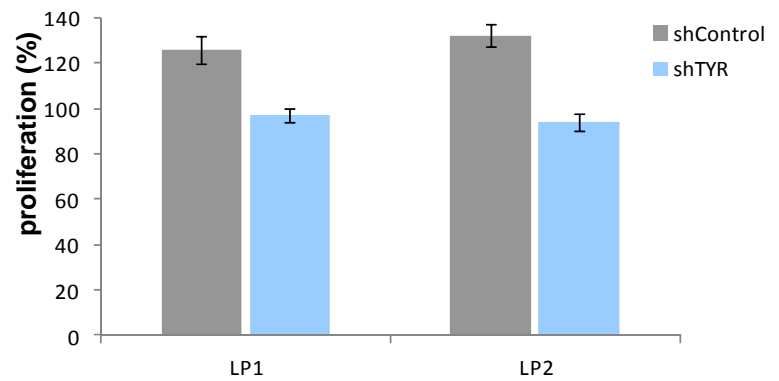


Figure 7C

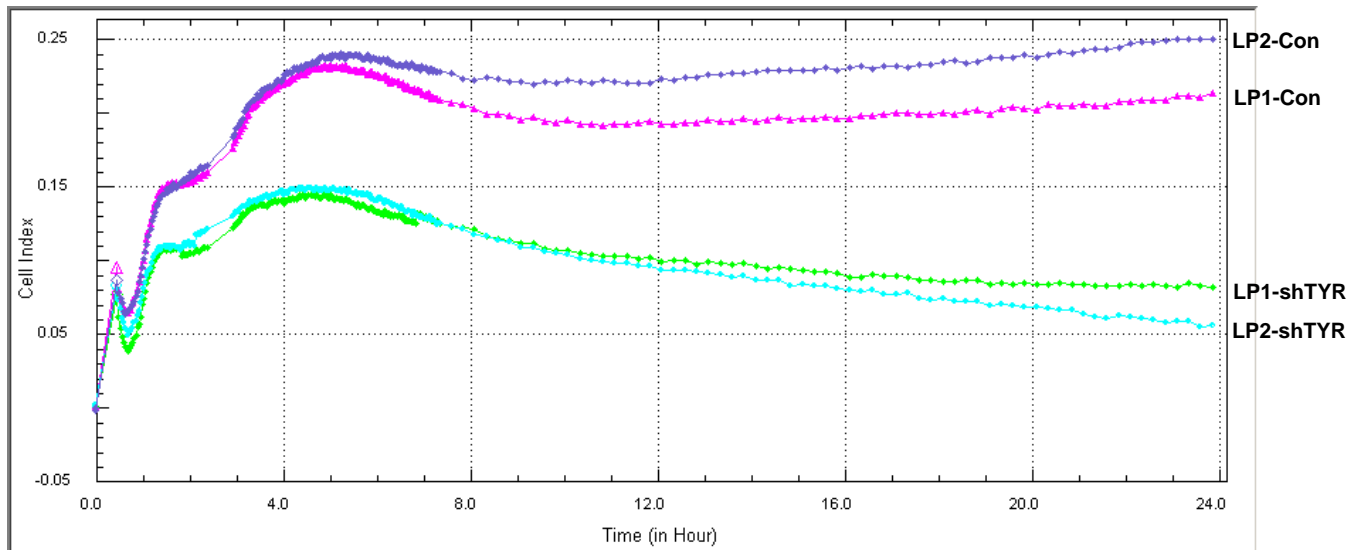


Figure 7: (A) The LP cells stably expressing shCon and shTyr were treated with tyrosine for 6 months. They were then seeded in 6-well plates, incubated for 7 days, harvested, propidium iodide labeled, and cell cycle analysis was performed. (B) After 6 months of tyrosine treatments, the LP-Con and LP-shTYR cells were seeded in 96-well plates and incubated for 7 days, after which the CellTiter-Blue proliferation assay was performed. The data showed that tyrosine treatments increased the proliferation of the LP-Control cells at a rate >20% of the untreated cells (100%), but it not alter the proliferation of the LP-shTYR cells. (C) An electrical impedance-based cell migration assay was performed using the LP-Con and LP-shTYR cells that were treated with tyrosine for 6 months. The data showed increased migration (measured as Cell Index) of the LP-Con cells, but not the LP-shTYR cells. The Cell Index curves for the specific cells are labeled at the right.